

# Effects of Ozone Exposure on Lipid Metabolism in Human Alveolar Macrophages

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Alveolar macrophages (AM) store arachidonic acid (AA), which is esterified in cellular phospholipids until liberated by phospholipase A<sub>2</sub> or C after exposure to inflammatory stimuli. After release, there can be subsequent metabolism of AA into various potent, biologically active mediators including prostaglandins and platelet-activating factor (PAF). To examine the possibility that these mediators may account for some of the pathophysiologic alterations seen in the lung after ozone (O<sub>3</sub>) exposure, human AM were collected by bronchoalveolar lavage of normal subjects, plated into tissue culture dishes, and the adherent cells were incubated with [<sup>3</sup>H]AA or [<sup>3</sup>H]lysoPAF. Human AM exposed to 1.0 ppm O<sub>3</sub> for 2 hr released 65 ± 12% more tritium, derived from [<sup>3</sup>H]AA, than paired, air-exposed controls into media supernatants. In other studies using a similar O<sub>3</sub> exposure protocol, there was also a significant increase in human AM prostaglandin E<sub>2</sub> production (2.0 ± 0.5-fold increase above air-exposure values, *p* < 0.01, *n* = 17). In additional studies, using a similar O<sub>3</sub> exposure protocol (1.0 ppm for 1 hr), there was also a significant increase in human AM PAF content (1.7 ± 0.2-fold increase above air-exposure values, *p* < 0.02, *n* = 5). These potent lipid mediators, originally derived from human AM, may play an important role in the mechanisms of O<sub>3</sub> lung toxicity.

## Introduction

Ozone (O<sub>3</sub>) is a major photochemical air pollutant that causes deleterious health effects on inhalation (1). This oxidant gas, formed by complex reactions of nitrous oxides with oxygen, can be found at concentrations exceeding 0.3 ppm in several U.S. areas, with ambient levels generally around 0.01–0.04 ppm. The lung is the main site of O<sub>3</sub>-induced toxicity, although extrapulmonary effects have been reported. Altered lung function, bronchial hyperresponsiveness, and a lung inflammatory response (measured as the presence of neutrophils and biochemical mediators of inflammation, e.g., AA metabolites, in bronchoalveolar lavage) have been reported in humans after O<sub>3</sub> exposure (2–4).

An important resident cell of the lung that may be affected by ozone is the alveolar macrophage. These cells are the predominant cell type within the alveolus and serve as the resident mononuclear phagocytes of the lung. Alveolar macrophages play an important role in immune and inflammatory processes be-

cause of the numerous mediators they secrete in response to phagocytic or inflammatory stimuli (5,6). Therefore, alveolar macrophages serve as the first line of host defense against inhaled organisms and soluble and particulate molecules. Ozone exposure has been shown to adversely affect host defense mechanism(s) of animals against certain infectious agents. Inhalation of O<sub>3</sub> has been reported to result in a significant increase in mortality of mice exposed to aerosols of inhaled microorganisms (7). It has been suggested that this effect may be attributable, at least in part, to perturbations in pulmonary alveolar macrophage immune defense mechanisms. Decreased phagocytosis, decreased superoxide anion production, decreased lysosomal enzyme release, and decreased interferon synthesis have been reported for alveolar macrophages after either *in vivo* or *in vitro* O<sub>3</sub> exposure (8–13). This observed toxicity of O<sub>3</sub> to alveolar macrophage functions may be due in part to the location of alveolar macrophages in the airway lumen, allowing greater exposure to O<sub>3</sub> compared to other lung cell types.

Metabolites of the membrane lipid constituents of alveolar macrophages represent an important class of macrophage mediators. Alveolar macrophages can store and metabolize the important lipid mediator arachidonic acid (AA). AA, a 20-carbon fatty acid, is usually esterified in cellular phospholipids in most cell types until liberated by phospholipase A<sub>2</sub> or C activity from membrane phospholipids. Once liberated, AA can serve as a source for the production of potent lipid mediators including cyclooxygenase and lipoxygenase products and platelet-activating factor (PAF).

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Metabolism of AA through cyclooxygenase initially generates the oxygenated product prostaglandin endoperoxidase  $G_2$ . This endoperoxide is enzymatically converted to another cyclic endoperoxide,  $PGH_2$ , by either cyclooxygenase activity or other peroxidases.  $PGH_2$  can be further metabolized to prostacyclin ( $PGI_2$ ) via prostacyclin synthetase activity; thromboxane  $A_2(TxA_2)$  by thromboxane synthetase catalysis; or other primary prostaglandins (PG) through enzymatic and nonenzymatic means ( $PGE_2$ ,  $PGF_{2\alpha}$ ). Cyclooxygenase activity can be stimulated by low concentrations of either hydroperoxides and/or lipid peroxides (generally 1–10  $\mu M$  depending on the cell type), whereas high concentrations (usually 10–100  $\mu M$ ) are inhibitory to activity. In addition, cyclooxygenase is a "suicide" enzyme, i.e., the enzyme is inactivated after conversion of a sufficient amount of substrate into product most probably through formation of an active oxygen species.

Conversion of free AA by the action of lipoxygenases results in the formation of unstable hydroperoxyeicosatetraenoic acids (HPETEs), generally the 5-, 12-, or 15- isomers in most cell types. These compounds are then metabolized either to their hydroxy- or dihydroxy-acid forms, e.g., 15-HETE, or to leukotrienes when 5-HPETE is converted to the unstable intermediate leukotriene  $A_4(LTA_4)$ . The 5-lipoxygenase pathway is a major pathway of AA metabolism in alveolar macrophages.  $LTA_4$  is converted either to  $LTB_4$  by enzymatic hydrolysis or to the sulfidopeptide leukotrienes ( $LTC_4$ ,  $LTD_4$ ,  $LTE_4$ ) via the addition and sequential cleavage of glutathione via the action of glutathione-S-transferase,  $\gamma$ -glutamyl transpeptidase, and dipeptidase. The leukotrienes  $C_4$ ,  $D_4$ , and  $E_4$ , collectively known as "slow-reacting substance of anaphylaxis" (SRS-A), cause bronchoconstriction via smooth muscle contraction and stimulate mucus secretion either individually or in the SRS-A complex.  $LTC_4$  and  $LTD_4$  produce coughing and chest tightness in both normal and asthmatic subjects. They also increase vascular permeability.  $LTB_4$  is a potent chemotactic agent for PMNs and eosinophils and may play a role in the recruitment of PMNs to inflammatory foci in the lung. It is unclear at present whether lipoxygenases are self-inactivated after metabolism of AA. Unlike cyclooxygenase, lipoxygenases are not known to possess the peroxidase activity for converting their hydroperoxy acid metabolites to the alcohol form.

PAF is synthesized and secreted in response to a variety of stimuli in several cell types, particularly those cells, such as the alveolar macrophage, that are involved in inflammatory and immune functions. PAF has now been identified as 1-O-alkyl-2-acetyl-sn-GPC (glycero-3-phosphocholine). Some of the bioactions of PAF include platelet aggregation, degranulation, migration, and superoxide production by PMN, and bronchoconstriction. The immediate precursor of PAF is lyso-PAF (in the "remodeling pathway," which is generally believed to be responsible for stimulus-mediated PAF synthesis that occurs in inflammatory cells such as the alveolar macrophage), which appears transiently after cell activation. Lyso-PAF is derived from preexisting cellular pools of 1-O-alkyl-2-acyl-sn-GPC through the action of a phospholipase  $A_2(PLA_2)$  to form 1-O-alkyl-2-lyso-GPC, which in turn is acetylated by an acetyltransferase to produce PAF (1-O-alkyl-2-acetyl-GPC). This first step of PAF synthesis, i.e., the cleaving of AA from alkyl-arachidonyl-GPC, thus releases two important precursor molecules, AA and lyso-PAF.

Thus, there are several potential mechanisms for the release of AA in alveolar macrophages. Depending on the type of AA release mechanism(s), a number of potent and important lipid mediators may be formed, which can play an important role in the inflammatory and physiologic response of the lung. We now report that exposure of human alveolar macrophages *in vitro* to  $O_3$  results in significant changes in alveolar macrophage lipid metabolism including release of AA, increased synthesis of  $PGE_2$ , and increased content of PAF.

## Materials and Methods

### Reagents

Common American Chemical Society-grade chemicals and reagents, HPLC-grade organic solvents, polypropylene and glass tubes, Whatman paper, pipettes, tissue culture disposables, and Scintinver LC scintillation cocktail were purchased from Fisher Scientific (Raleigh, NC). Biochemicals, including enzymes and enzyme reagents, Tris, HBSS, bovine serum albumin, EGTA, formyl-methionyl-leucyl-phenylalanine (FMLP), A23187, phospholipids, and platelet-activating factor, Triton X 100, siliconizing reagent, and acid phosphatase reagents were all obtained from Sigma Chemical Company (St. Louis, MO). [ $^3H$ ]AA was obtained from NEN Dupont (Boston, MA). [ $^{14}C$ ]PAF and [ $^3H$ ]LysoPAF were purchased from Amersham (Arlington Heights, IL).

### Ozone Exposure System

Cells cultures were exposed to predetermined concentrations of ozone or air in 35- or 60-mm tissue culture polystyrene petri dishes using an *in vitro* ozone chamber system (Fig. 1) and consisting of two plexiglass and stainless-steel chambers (15 L in capacity) through which filtered room air at 37°C, with or without  $O_3$ , was passed at a flow rate of 7.5 L/min. Separate input lines carried humidified air containing  $CO_2$  into the chambers to maintain a 5%  $CO_2$  atmosphere as regulated by 2 CH/P  $CO_2$  Analyzers (Forma Scientific, Marietta, OH). The chambers were mounted on rocking platforms (Bellco Glass, Vineland, NJ), which permitted rocking of the petri dishes during exposure. Chambers and platforms were enclosed in 37°C incu-

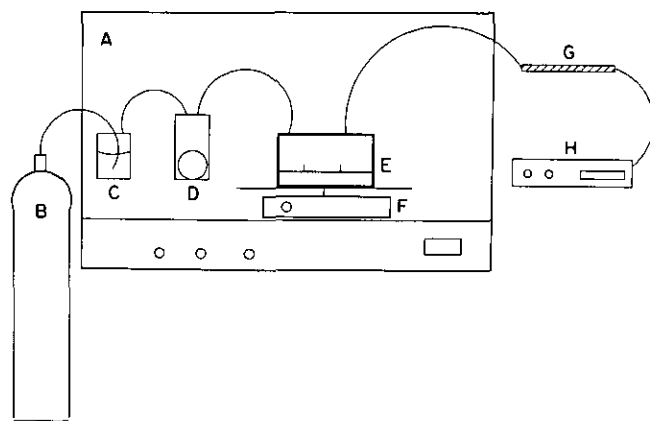


FIGURE 1. *In vitro* ozone exposure system. A, temperature-controlled incubator; B, zero-grade air; C, humidifier; D, ozone generator; E, plexiglass and steel chamber; F, rocker platform; G, condenser; H, ozone analyzer.

bators (Forma Scientific, Marietta, OH). Ozone was generated by passing the humidified air over a UV lamp; the brightness was regulated by partially covering the lamp with a steel tube until the desired  $O_3$  concentration was reached. Ozone concentrations were determined by sampling the chamber atmosphere, dehumidifying it with a condenser, and passing the air into a Model 1003-AH Ozone Analyzer (Dasibi, Glendale, CA), which was periodically calibrated by instrumentation traceable to the National Bureau of Standards. All lines and fittings in the system were Teflon, glass, or stainless steel. The pH of phosphate-buffered saline (PBS) or Hank's buffered salt solution was found to be unchanged after 2 hr of exposure to 1.0 ppm  $O_3$ .

### Human Alveolar Macrophages

Healthy, nonsmoking male volunteers 18–35 years of age underwent fiberoptic bronchoscopy and bronchoalveolar lavage to obtain normal human alveolar macrophages for use in these experiments. Each subject was remunerated and signed a statement of consent after being informed of the purpose, procedure, and risks of the bronchoalveolar lavage protocol. The procedure was approved by the Committee on the Rights of Human Subjects of the University of North Carolina School of Medicine.

Before bronchoscopy, all subjects were premedicated with 0.5 mg of intravenous atropine. The posterior pharynx was anesthetized by gargling with a 4% lidocaine solution in saline and a lubricating jelly containing 2% lidocaine was placed in the nostril through which the bronchoscope (BF-1T10, Olympus, New Hyde Park, NY) was passed. In addition, some subjects were administered 25–50 mg of Demorol intravenously. Lidocaine was injected through the bronchoscope at the level of the vocal cords, the carina, and more distal airway bifurcations to decrease coughing. A maximum of 20 mL of 2% lidocaine was used. For the lavage, the bronchoscope was wedged in a segmental or subsegmental bronchus of the lingula, and 50 mL of sterile saline at room temperature was slowly injected through the bronchoscope. The saline was immediately aspirated into a 50 mL sterile polypropylene tube, which was then placed on ice. A total of six washes were performed for a total of 300 mL of saline instilled. This procedure was then repeated in the right middle lobe with an additional 300 mL of saline. Approximately  $30 \times 10^6$  total viable alveolar macrophages are recovered from a typical subject, with a recovery of the injected saline of approximately 75%. The viability of the recovered cells exceeded 85% by trypan blue exclusion.

Immediately after the procedure, the lavage fluid containing the cells was centrifuged at 350g for 10 min at 4°C. Cells were then pooled and washed twice in RPMI-1640 supplemented with 0.025% gentamycin. Between the two washes, cells were counted in a hemocytometer and adjusted to  $1 \times 10^6$  viable macrophages per milliliter of RPMI 1640. Cell differentials were done on cytocentrifuged slides prepared at 700 rpm for 5 min and stained with a modified Wrightstain (Leukostat Solution, Fisher Scientific, Fairlawn, NJ). A typical differential count yielded 88% alveolar macrophages, 11% lymphocytes, and 1% polymorphonuclear leukocytes.

### Radiolabeling of Cells

Alveolar macrophages, in culture flasks, were radiolabeled with 1  $\mu$  Ci/mL of either [ $^3$ H]LysoPAF or [ $^3$ H]AA for 30 min–5

hr in RPMI with 2% fetal calf serum. After labeling, the cells were washed three times with PBS and then 1.5 mL PBS with calcium, magnesium, and glucose (1 mg/mL) was added and the cells were exposed to either air,  $O_3$ , or the calcium ionophore A23187 (10  $\mu$ M). Approximately 70% of [ $^3$ H]lysoPAF in the medium was taken up by the cells by 30 min of incubation and 50% of the [ $^3$ H]AA was taken up after 4 hr.

### Extraction of Cellular Lipids

Reactions and cell exposures were terminated by scraping the cells from the dishes with a rubber scraper and transferring them to a polypropylene tube containing chloroform, rinsing the dish with methanol, and pooling the rinse with the chloroform/cell mix. The proportions of solvents were always 0.8:1.0:2.0 aqueous:methanol:chloroform, as described by Folch et al. for the extraction of lipids from tissues (23). In some cases, the chloroform contained a few hundred disintegrations per minute of [ $^{14}$ C]PAF as an internal standard. After mixing, the tubes were centrifuged at 500g for 2 min to separate the phases and the chloroform phase was removed with a pasteur pipette and placed in a siliconized disposable glass tube. A second extraction was performed on the aqueous/methanol phase with an additional two volumes of chloroform, and the chloroform phases were pooled and evaporated in a 37°C heating block under a stream of  $N_2$ . The resulting lipid residue was redissolved in a small volume of chloroform for HPLC or TLC.

### High-Performance Liquid Chromatography

Two different HPLC methods were employed, one specifically designed for the separation of phospholipids and another for the separation of AA metabolites. The instrumentation used was the same for all methods and consisted of two Waters 510 HPLC Pumps, a U6K manual injector, a System Interface Module, a 490E Multiwavelength Detector, a NEC APC IV computer, and the 820 Maxima software program (Waters Millipore, Millford, MA). Detection of radioactivity in the HPLC effluent was monitored in a CR Flow ONE Beta Radioactive Flow Detector with a DTK Data 1000 computer (Radiomatic Instruments, Tampa, FL). Scintiverse LC liquid scintillation cocktail was used at a mixing ratio of 3:1.

### Phospholipid Separation

The separation method is described in greater detail in another publication (14). The method uses two 25 cm  $\times$  4.8 mm cyanopropyl columns in series, connected with a 10-cm length of 0.010 in of stainless-steel tubing and protected by an LC-CN guard column cartridge. The mobile phase, solvents A and B, consisted of 100% acetonitrile and 80% acetonitrile/20% 5 mM sodium acetate (pH 5.0), respectively, and were filtered and degassed under vacuum before use. Solvent B was prepared daily to prevent precipitation of salts. The mobile phase and columns were kept at 60°C via a circulating water bath to facilitate the separation of the acidic phospholipids and reduce the back pressure in the system. A gradient was used at a flow rate of 2 mL/min, as follows: from 0 to 5.0 min 10% B, from 5.1 to 12.0 min increasing to 75% B, from 12.1 to 30.0 min maintaining 75%

B, from 30.1 to 30.2 min decreasing to 10% B, and from 30.3 to 45.0 re-equilibrating at 10% B. The columns were regenerated regularly with 50 mL of 30% acetonitrile, followed by 200 mL of 100% acetonitrile, and the guard column cartridges were replaced periodically. Peaks of interest isolated with this HPLC system were evaporated under  $N_2$ , redissolved in chloroform, methanol and water, and extracted. The chloroform phases were then dried under  $N_2$  and redissolved in the desired solvent.

## HPLC Analysis of [ $^3H$ ]Arachidonic Acid Metabolites

Media were analyzed using a previously published HPLC procedure (15). Just before analysis, samples were brought to room temperature, mixed (30 sec), and centrifuged at 2000g for 6 min. An aliquot (750  $\mu$ L) of the supernatant was then injected into the HPLC system employing an Alltech Ultrasphere ODS 5  $\mu$ m reverse-phase column (Rainin Instrument Co., Woburn, MA) preceded by a precolumn filter (0.5  $\mu$ m frit; Upchurch Scientific, Oak Harbor, WA). Pump A delivered a water/methanol/acetic acid (9/1/0.01 v/v/v) solution, pH 5.05, prepared by titration with concentrated  $NH_4OH$ ; pump B delivered 100% methanol to the system. With a flow rate of 1.1 mL/min throughout the run, a gradient elution was utilized with linear changes in eluant composition at the following time points: 0 min, 47% pump A; 27 min, 40% pump A; 52 min, 27% pump A; 74 min, 0% pump A; 101 min, re-equilibration to initial conditions for 15 min. Metabolite peaks were identified as eicosanoids based on the retention time compared to externally applied authentic standards. Radioactivity associated with peaks were corrected for baseline radioactivity.

Radioimmunoassay (RIA) of AA metabolites was performed using kits purchased from either NEN Dupont (Boston, MA), Amersham (Arlington Heights, IL), or Advanced Magnetics (Boston, MA).

## Thin Layer Chromatography

TLC was used to isolate, quantify, and identify PAF according to the method described by Chilton et al. (16). The chromatography was carried out on silica gel 60 plates that were heat activated at 120°C for at least 1 hr before use. Samples were spotted under a stream of warm air from a blow drier 2 cm from the bottom of the plate in 20–50  $\mu$ L of chloroform with a microliter syringe. The plate was reheated at 120°C for 3 min, cooled to room temperature, and developed to within 1 cm from the top. The development tank was lined with 3MM Whatman paper and equilibrated with a solvent mix consisting of 100 mL of chloroform, 50 mL of methanol, 16 mL of glacial acetic acid, and 8 mL of water. After development, the plate was allowed to air dry under a fume hood and the lipid bands were visualized in a tank containing sublimed  $I_2$  crystals. For quantification of radioactive species, the bands were scraped into scintillation vials, 1 mL of methanol:water 1:1 and 3 mL of Scintiverse LC were added, and the vials were counted for 5 min in a TRI-CARB 1500 Liquid Scintillation analyzer (Packard, Sterling, VA), with a quench-correcting program capable of discriminating between  $^3H$  and  $^{14}C$  disintegrations per minute.

Data were analyzed using *t*-tests for paired or unpaired variates. All data are expressed as means  $\pm$  standard error of the mean.

## Results and Discussion

Human alveolar macrophages were plated into tissue culture dishes and the adherent cells (> 95% plating efficiency) were incubated with [ $^3H$ ]AA for 1–24 hr. A 4-hr period is sufficient to equilibrate [ $^3H$ ]AA into cellular lipid pools within alveolar macrophages as assessed by HPLC (Table 1). There were no major changes in the tritium associated with each cellular pool after 2 hr of [ $^3H$ ]AA labeling, with the possible exception of the radioactivity associated with triglycerides, which decreased from containing 29% of the tritium at 1 hr to 15% at 24 hr and an increase in the combined PE + LPE pool from 5 to 28%. Phosphatidylcholine was the major reservoir of [ $^3H$ ]AA at 1–16 hr (> 24% at all time points sampled).

After incorporation of [ $^3H$ ]AA into cellular pools, human alveolar macrophages were washed to remove unincorporated label, 0.5 mL phosphate-buffered saline with glucose (1 mg/mL; PBS-glucose) was added, and then alveolar macrophages were exposed to air or 1.0 ppm  $O_3$  for 2 hr. After exposure, media was centrifuged and an aliquot counted for radioactivity. Human alveolar macrophages exposed to  $O_3$  released  $65 \pm 12\%$  more tritium, derived from [ $^3H$ ]AA, than paired air-exposed controls into media supernatants (Table 2). Human peripheral blood monocytes were prelabeled with [ $^3H$ ]AA and exposed to  $O_3$  in a similar manner. This cell type had a similar response as the alveolar macrophage (Table 2). Both cell types remained viable throughout the exposures with no difference in viabilities (> 90% trypan blue exclusion) between  $O_3$ -exposed and air-exposed cells. Approximately 10% of the cells detached during either air or  $O_3$  exposure. In a separate study, there appeared to be a continuous increase in release of tritium over the 2-hr exposure period in the ozone group ( $1.42 \pm 0.39\%$  at 15 min;  $2.03 \pm 0.32\%$  at 30 min;  $3.14 \pm 1.21\%$  at 160 min; and  $3.58 \pm 1.13\%$  at 20 min) compared to the air-exposed cells. The values at the 30- and 120-time points were significantly higher than those obtained in paired cultures exposed to air alone for similar time periods ( $p < 0.05$ ;  $n = 4-5$ ).

**Table 1. Distribution of [ $^3H$ ]Arachidonic acid in lipid pools with human alveolar macrophages.**

Time of incubation with [ $^3H$ ]AA, hr	n	$^3H$ [AA] distribution in lipid classes, % total lipid classes							Free AA
		PC	PI	PE + LPE	SM + LPC	PG	PS	TG	
1	5	31	12	5	6	5	< 0.2	29	5
2	4	33	14	8	7	1	< 0.2	24	5
4	4	37	14	13	10	1	< 0.2	16	3
16	5	29	12	23	10	0.7	< 0.2	13	3
24	5	24	9	28	8	2	< 0.2	15	6

Abbreviations: PC, phosphatidylcholine; PI, phosphatidylinositol; TG, triglycerides; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; LPC, lysophosphatidylcholine; SM, sphingomyelin; AA, arachidonic acid; LPE, lysophosphatidylethanolamine.

**Table 2. Effect of  $O_3$  on the release of tritium by human monocytes and alveolar macrophages.\***

Exposure, 2 hr	% $^3H$ released	
	Alveolar macrophages	Blood monocytes
Air	$3.4 \pm 0.6$ (22)	$9.1 \pm 3.4$ (5)
1.0 ppm $O_3$	$5.6 \pm 1.0$ *(22)	$14.8 \pm 5.2$ *(5)

\*Tritium derived from cellular [ $^3H$ ]Arachidonic acids. Values are means  $\pm$  SEM; n in parentheses.

\*Significant difference from air-exposed value,  $p < 0.05$ .

HPLC analysis of media supernatants from human  $O_3$ -exposed alveolar macrophages revealed that the released radioactivity was associated with HETEs, free AA, and the highest radioactivity was associated with a polar peak that eluted with a similar retention time as  $O_3$ -exposed AA. This lack of change in eicosanoid content compared to our previously published data in rat macrophages, using similar exposure and separation methods (17), may have been due to an insufficient number of alveolar macrophages to synthesize detectable AA metabolites ( $< 5 \times 10^6$  human macrophages were used per exposure group compared to  $> 5 \times 10^6$  rat macrophages used previously), degradation of formed AA metabolite, or AA itself into other derivatives, and/or methodological reasons. Therefore, a more sensitive technique, radioimmunoassay, was used to determine eicosanoid concentrations in media supernatants of alveolar macrophages exposed to air or  $O_3$ . This technique can detect eicosanoids at approximately 100 pg/mL concentrations. Human alveolar macrophages were cultured as previously described, washed to remove serum, 0.5 mL PBS-glucose was added, and the cells were exposed to air or  $O_3$  (0.1–1.0 ppm for 2 hr). After exposure, medium was collected, centrifuged, and medium supernatants analyzed by radioimmunoassay (RIA) for specific metabolites. The results are shown in Table 3. Alveolar macrophages exposed to  $O_3$  produced more  $PGE_2$  than paired, air controls in a concentration-dependent manner, with a significant 2.03-fold increase observed in the 1.0-ppm exposed cultures ( $p < 0.05$ ). No differences were observed in the formation of  $TxB_2$  or  $LTB_4$  between the air-exposed and  $O_3$ -exposed alveolar macrophages.

To further determine whether alterations in AA metabolism were present after  $O_3$  exposure, immediately after exposure to air or  $O_3$  (0.1–1.0) for 2 hr, the cells were washed and then incubated (at 37°C with 5%  $CO_2$ ) with 1.0 mL serum-free RPMI containing 10  $\mu$ M calcium ionophore A23187 (in 0.13% DMSO) for 30 min. The medium was collected, centrifuged, and supernatants analyzed by RIA for eicosanoid content. Results are shown in Table 4. These data show that  $O_3$ -exposed

alveolar macrophages still could produce eicosanoids, similar to air-exposed cultures in response to calcium ionophore incubation. These data suggest that the observed  $O_3$ -dependent increase in alveolar macrophage  $PGE_2$  synthesis was not via a calcium-dependent mechanism.

Ozone exposure has been shown to alter AA metabolism in the lung. A 10-fold increase in AA was found in bronchoalveolar lavage fluid of rats exposed to 1.1 ppm  $O_3$  (24). Seltzer and associates found increased levels of prostaglandins  $E_2$  and  $F_{2\alpha}$  and thromboxane  $B_2$  (a product of thromboxane  $A_2$ ) in bronchoalveolar lavage fluid of subjects exposed to 0.4 ppm  $O_3$  for 2 hr (2). A 2-fold increase in prostaglandin  $E_2$  in the lavage fluid of subjects exposed to 0.4 ppm for 2 hr was reported by Koren et al. (3). Further evidence of the significance of eicosanoids in  $O_3$  toxicity was provided by studies reporting protection against  $O_3$ -induced decreases in functional volume capacity and forced expiratory volume in 1 sec in subjects administered the cyclooxygenase inhibitor indomethacin before exposure to 0.35 ppm  $O_3$  for 1 hr (18). *In vitro* studies have also shown AA and eicosanoid release from lung cells exposed to  $O_3$ . Bovine endothelial cells exposed to 0.3 ppm  $O_3$  for 2 hr were shown to release AA (24), whereas bovine epithelial cells exposed to 0.1 ppm  $O_3$  for 2 hr released increased amounts of prostaglandin  $F_{2\alpha}$  (19).

The effect of *in vitro*  $O_3$  exposure on eicosanoid release from alveolar macrophages has also been studied. Rabbit alveolar macrophages released prostaglandin  $E_2$  and other cyclooxygenase products when exposed to 0.3 and 1.2 ppm  $O_3$  for 2 hr (20). A 3.3-fold increase in AA release was found in rat alveolar macrophages exposed to 1.0 ppm  $O_3$  for 2 hr. These authors also found increases in thromboxane  $B_2$  and leukotrienes  $B_4$ ,  $C_4$ , and  $D_4$  (17). These studies indicate that  $O_3$  can alter the amounts of AA metabolites biosynthesized as well as increase the release of the parent compound, AA. Reports indicating  $O_3$ -induced alterations of AA product formation suggest that these alterations are due to modifications in enzymatic activities (17).

As reviewed, macrophages can respond to a variety of stimuli by production of another lipid metabolite, PAF. The effects of a 2-hr *in vitro* exposure to human alveolar macrophages, prelabeled with precursor [ $^3H$ ]lysoPAF, are shown in Table 5. No changes in viability were seen in cells exposed to 1.0 ppm  $O_3$  compared to air-exposed cells for 60 min. As shown, human alveolar macrophages obtained from healthy subjects, radiolabeled and exposed in culture to 1.0 ppm  $O_3$  for 60 min, had a 1.7-fold significant increase in the level of [ $^3H$ ]PAF compared to human alveolar macrophages exposed to air alone ( $p < 0.002$ ,  $n = 5$ ). In contrast, human alveolar macrophages incubated with 10  $\mu$ M A23187 for 60 min had nonsignificant increases in [ $^3H$ ]PAF levels (1.1  $\pm$  0.1-fold,  $n = 3$ ) compared to vehicle alone.

PAF is derived from preexisting cellular pools of 1-*O*-alkyl-2-arachidonyl-FPC through the action of a phospholipase

**Table 3. Human alveolar macrophage eicosanoid production in response to  $O_3$  exposure (2 hr).**

$O_3$ concentration, ppm	Eicosanoid synthesis, % air exposure value <sup>a</sup>		
	$PGE_2$	$TxB_2$	$LTB_4$
0.1	125 $\pm$ 13 (7)	91 $\pm$ 8 (7)	—
0.3	168 $\pm$ 22 (9)*	64 $\pm$ 7 (9)	—
1.0	203 $\pm$ 34 (17)*	69 $\pm$ 13 (12)	89 $\pm$ 34 (4)

Abbreviations:  $PGE_2$ , prostaglandin  $E_2$ ;  $TxB_2$ , thromboxane  $B_2$ ;  $LTB_4$ , leukotriene  $B_4$ .

<sup>a</sup>Data are shown as means  $\pm$  SEM;  $n$  in parentheses.

\*Significant difference from paired, air control value,  $p < 0.05$ .

**Table 4. The effect of  $O_3$  on human alveolar macrophage eicosanoid synthesis in response to calcium ionophore A23187.<sup>a</sup>**

$O_3$ concentration, ppm	Eicosanoid synthesis, % of air exposure		
	$PGE_2$	$TxB_2$	$LTB_4$
0.1	164 (2)	96 (2)	—
0.3	175 (2)	61 (2)	28 (2)
1.0	159 $\pm$ 33 (5)	202 $\pm$ 119 (5)	96 $\pm$ 7 (7)

Abbreviations:  $PGE_2$ , prostaglandin  $E_2$ ;  $TxB_2$ , thromboxane  $B_2$ ;  $LTB_4$ , leukotriene  $B_4$ .

<sup>a</sup>Calcium ionophore, 10  $\mu$ M, 30 min. Data are shown as means  $\pm$  SEM;  $n$  in parentheses.

**Table 5. Effect of ozone exposure (1.0 ppm  $\times$  1 hr) on human alveolar macrophage platelet-activating factor (PAF) synthesis.<sup>a</sup>**

PAF production, dpm $\times 10^2$	$O_3$ /air	$n$
91.8 $\pm$ 84.8*	1.7 $\pm$ 0.2	5

<sup>a</sup>The results are shown as means  $\pm$  SEM and expressed as number of disintegrations per minute (dpm) of [ $^3H$ ]PAF in  $O_3$ -exposed cultures. All data were adjusted for recovery of a [ $^{14}C$ ]PAF internal standard.

\*Significant difference from air control values,  $p < 0.02$ .

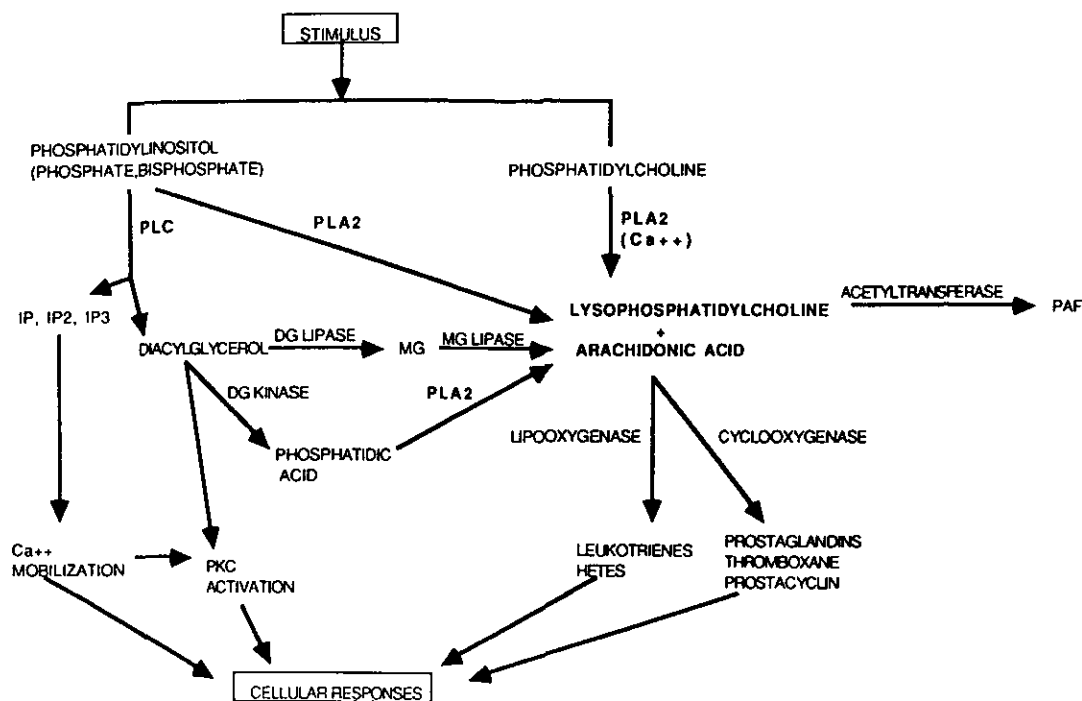


FIGURE 2. Schematic diagram of cellular lipid interactions.

A<sub>2</sub> to form 1-*O*-alkyl-2-lyso-GPC, which is in turn acetylated by an acetyltransferase to produce PAF (1-*O*-alkyl-2-acetyl-GPC). There is evidence that the PAF acetyltransferase enzyme can be activated in some stimulated cells through a biochemical mechanism involving protein phosphorylation by serine/threonine kinases and suggest a role of protein kinase C, a Ca<sup>2+</sup>-dependent and phospholipid-dependent protein kinase, which is a pivotal regulatory element in signal transduction. PAF is degraded by PAF-acetylhydrolase, an enzyme that is highly selective for phospholipids with short acyl groups at the *sn*-2 position and thus catalyzes the inactivation of PAF by removal of the acetate moiety from PAF to form alkyllyso-GPC, which is biologically inactive. Interestingly, it has recently been shown that oxidized phospholipids, such as those that could occur in cells after O<sub>3</sub> exposure, are degraded by the PAF acetylhydrolase (25).

The metabolism and biologic effects of these two classes of lipid mediators, eicosanoids and PAF, may also be interrelated. The release of AA from the *sn*-2 position of phospholipids is postulated to be the first step in the production of cyclooxygenase and lipoxygenase products as well as PAF. Alveolar macrophages are rich in esterified AA, and the release appears to be calcium mediated, including phospholipase A<sub>2</sub>-mediated deacylation, phospholipase C action, and enzymatic processes governing formation of diacyl glycerol, cardiolipin, and phosphocholine/ethanolamine from phosphatidylcholine (PC) and phosphatidylethanolamine (PE). However, there is a complex pattern of AA turnover in phospholipids of alveolar as shown in Figure 2. In unstimulated cells, AA is predominantly incorporated into PI and the diacyl species of PE and PC and then mobilized to the ether-linked species of PE and PC. In stimulated cells, several phospholipid species (primarily phosphoinositides and ether- and ester-linked species of PC) serve as sources of AA for oxygenation into various bioactive mediators. Thus, in stimulated

cells both phospholipase A<sub>2</sub> and phospholipase C are activated. Of the total label lost from PC in response to inflammatory stimuli such as lipopolysaccharide or opsonized zymosan, half is derived from the 1-alkyl-linked species and the rest from 1-acyl-linked species. The phospholipase A<sub>2</sub> enzyme can hydrolyze alkyl-linked PC, providing a substrate for PAF production. The phospholipase C can activate phosphoinositide biphosphate to produce diacylglycerol and inositol phosphates. These reactions may involve specific receptors that link G-proteins, protein kinases, phospholipase A<sub>2</sub> and phospholipase C, phosphatidylinositol turnover, cytosolic Ca<sup>2+</sup>, Ca<sup>2+</sup>-dependent effectors such as calmodulin, diacylglycerol, and monoglycerol lipase, to the production of bioactive AA metabolites and PAF. Thus, there are several potential mechanisms for the release and subsequent metabolism of AA in alveolar macrophages in response to O<sub>3</sub>.

In summary, our results demonstrate that O<sub>3</sub> exposure results in a concentration-dependent release of AA from cell membranes of human alveolar macrophages with subsequent increased synthesis of several important lipid mediators including PGE<sub>2</sub> and PAF. These lipid mediators formed in response to O<sub>3</sub> may play an important role in the inflammatory and physiologic response of the lung to O<sub>3</sub> exposure. Based on the pathways depicted in Figure 2, these data suggest that O<sub>3</sub> may produce cellular responses through one or more enzymatic pathways that may be receptor mediated. Future work directed at the cellular processes regulating the biosynthesis and secretion of potent lipid mediators will further the understanding of the response of the lung to inhaled toxicants such as O<sub>3</sub>.

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